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Review

A systems perspective on brown adipogenesis and metabolic activation

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Summary

Brown adipocytes regulate energy expenditure via mitochondrial uncoupling. This makes these fat cells attractive therapeutic targets to tackle the burgeoning issue of obesity, which itself is coupled to insulin resistance, type 2 diabetes, cardiovascular and fatty liver disease. Recent research has revealed a complex network underlying brown fat cell differentiation and thermogenic activation, involving secreted factors, signal transduction, metabolic pathways and gene regulatory components. Given that brown fat is now reported to be present in adult humans, it is desirable to harness the knowledge from each network module to design effective therapeutic strategies. In this review, we will present a systems perspective on brown adipogenesis and the subsequent metabolic activation of brown adipocytes by integrating signaling, metabolic and gene regulatory modules with a specific focus on known 'druggable' targets within each module.

Keywords: Brown adipogenesis, gene regulatory network, metabolism, signal transduction.

Introduction

With the increase in the global incidence of obesity and its associated pathologies, collectively termed the metabolic syndrome, there is renewed interest in exploring pathways that modulate energy balance in mammalian systems (1). Disproportional energy intake over expenditure leads to energy imbalance, excessive fat storage in the white adipose tissue (WAT), and disturbance of whole body homeostasis, igniting intense investigation into pathways that would enhance energy homeostasis (2,3). The latter is in part mediated by fat depots via secretion of cytokines and interaction with various organs of the body. WAT, known as an endocrine-type storage organ, is capable of secreting adipokines such as leptin and adiponectin (4), and communicating with different organs such as the brain, muscle and liver to regulate energy homeostasis as well as the immune response. Excessive energy intake leads to WAT hyperplasia (increase in the number of mature adipocytes) and/or hypertrophy (adipose tissue expansion). Given its status as an energy reservoir, WAT has therefore been the primary target to combat obesity. However, results have been discouraging due to lipid divergence, leading to the accumulation of lipids in other organs such as skeletal muscle, kidney and liver, ultimately causing the development of metabolic disorders. For instance, in response to the adipocyte-specific deletion of peroxisome proliferator-activated receptor gamma (*Pparg*), severe lipoatrophy is observed in mice, but the mass of *Pparg* null mice and their wild type littermates is similar, with the *Pparg* null mice demonstrating lipid divergence and afflictions such as fatty liver, diabetes and severe insulin resistance (5). This underscores the importance of designing effective therapies that lack adverse systemic side effects. While dietary restriction and exercise have a positive impact on adipose mass leading to diminished fat storage (6), novel strategies to improve whole body homeostasis and restore metabolic balance are being explored to aid metabolically challenged patients. With the recent positron emission tomography – computed tomography-based (PET-CT) discovery of metabolically active brown fat and recruitable brown-in-white fat depots in adult humans (7), current work in the field has turned to

explore ways to maximize energy expenditure via the brown adipose tissue (BAT) to combat excessive fat accumulation and restore energy homeostasis.

The presence of a second type of adipose tissue, BAT, has been originally described both in rodent models and in human infants, (8), as the thermogenic activity of BAT is essential for the endurance of small mammals in cold environments. A brown adipocyte is characterized by densely packed, iron-rich mitochondria, rendering it brown in color, and is capable of converting free fatty acids into heat via the action of the uncoupling protein 1 (UCP1) in response to various stimuli, most notably cold and diet (8). Apart from classical brown fat, WAT also harbors pockets of UCP1-expressing cells with thermogenic capacities that arise in response to various stimuli (9). These adipocytes are known as beige or brite (brown-in-white) or recruitable BAT (10) and display similar features to BAT, such as high mitochondrial content, multilocular lipid droplets and expression of a key set of brown fat marker genes (*Ucp1*, *Cidea, Pgc1a*) despite originating from different cellular lineages (11). Classical brown fat cells develop from pluripotent precursor cells in the somites that express transcription factors (TFs) such as PAX7, Homeobox protein engrailed-1 (EN1) and Myogenic factor 5 (MYF5). These TFs later determine the development of these precursor cells either into brown fat cells, skeletal myocytes, dorsal dermis or a subset of white adipocytes (12). On the other hand, lineage-tracing analysis showed that beige fat cells, together with another subset of white adipocytes, originate from *Myf5*-negative populations of progenitors (13) and express characteristic genes such as those coding for cluster of differentiation-137 (*Cd137*), T-box 1 (*Tbx1*) and transmembrane protein 26 (*Tmem26*) (14). Interestingly, a significant portion of the brown fat from adult humans that is present in the neck and supraclavicular regions has the molecular characteristics of beige (or recruitable) BAT rather than classical BAT (14,15). This is unlike the interscapular depots of human infants that have been shown to resemble classical BAT found in rodents using high resolution imaging, as well as histological and biochemical analyses (16). To design effective therapies promoting BAT activity in metabolically challenged human subjects, it is important to have a thorough understanding of the molecular mechanisms underlying brown fat cell development and activity. This review is an attempt to integrate the signaling, metabolic and transcriptional modules governing brown fat differentiation and metabolic activation with an additional focus on the white to brown axis. We envision that this network-based perspective will contribute to the identification of unexplored pathways or incomplete mechanistic details regarding key regulators and signaling molecules, aiding in the development of targeted therapeutic approaches.

Part 1: Metabolic activity of brown adipose tissue

Uncoupling protein 1, the core player in brown adipose tissue activation

The process of 'uncoupling' substrate oxidation from adenosine triphosphate (ATP) synthesis, i.e. thermogenesis, occurs in brown adipocytes and is catalyzed by the mitochondrial protein UCP1 (8). UCP1 is a multi-pass mitochondrion inner membrane protein and member of the mitochondrial carrier protein family (SLC25) (17–20). *Ucp1* null mice show little resistance to cold and are not capable of sustaining their body temperature during prolonged cold exposure, suggesting a crucial role for UCP1 in cold-induced thermogenesis (21). Thermogenesis at the molecular level begins when free long chain fatty acids (LCFAs) liberated from the abundant multilocular fat droplets within the brown adipocyte are activated by acyl-CoA synthase to acyl CoAs and transferred into the mitochondria. This results in beta-oxidation of LCFAs (now acyl-CoAs), which, in combination with the Krebs cycle, produces the reduced electron carriers, Flavin adenine dinucleotide (FADH2) and Nicotinamide adenine dinucleotide (NADH). The latter are subsequently oxidized within the respiratory chain, which causes the protons to be pumped out of the mitochondria, ultimately resulting in the formation of a proton-motive force. UCP1 then mediates the influx of protons back into the mitochondrial matrix without ATP being synthesized. Rather, the energy stored in the proton-motive force is liberated as heat (8,22). More specifically, the mechanism by which UCP1 transports H+ ions has recently been elucidated using the patch clamp technique. The latter allowed direct measurement of UCP1 currents in the native inner membrane of BAT mitochondria, demonstrating that UCP1 is a symporter of LCFAs/H^+. Thus, LCFAs serve as primary substrates that associate with UCP1 via hydrophobic interactions and enable UCP1 to transport H+ ions, thereby mediating thermogenesis (23).

Part 2: Known therapeutic interventions in humans

Non-drug-based approach

Methods that are employed to induce BAT development and activation, aiming to improve whole body energy homeostasis, include the following:

Cold exposure

Cold exposure, to our knowledge, is the most efficient approach to induce BAT development and activation (24,25), and it does so via the sympathetic nervous system (SNS) as well as the norepinephrine (NE)-dependent signal transduction pathway (Fig. 1). Numerous studies showed that the effects of chronic cold exposure are compelling and significantly improve systemic metabolic homeostasis, including insulin sensitivity as well as glucose and lipid profiles (24–27).

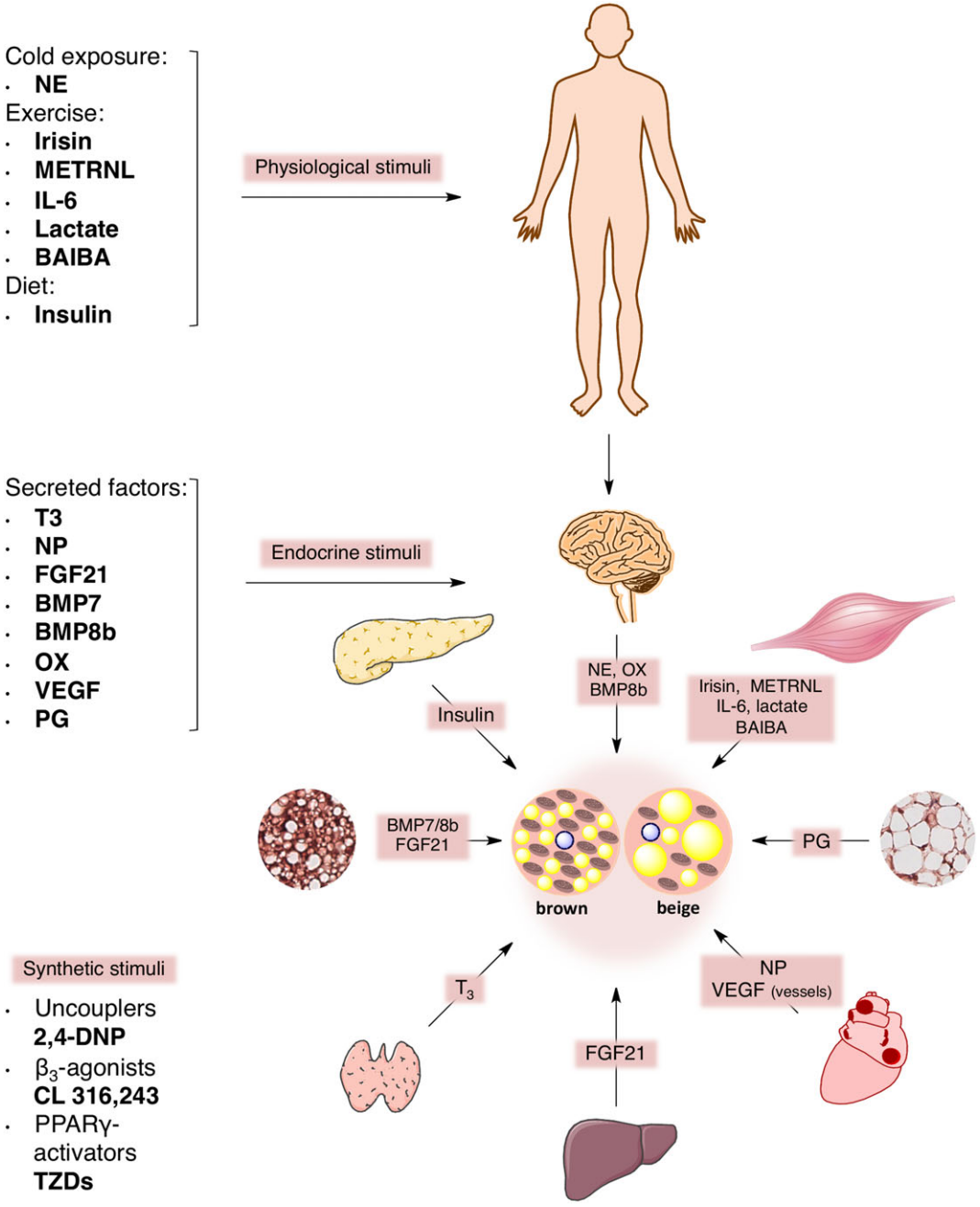

Figure 1 Schematic representation of factors that promote development and activity of brown and beige adipocytes. Shown are factors with therapeutic potential as BAT potentiators. Physiological stimuli originating at the systemic level can be divided into 1) cold exposure-derived factors namely norepinephrine (NE) that is secreted by the SNS; 2) exercise-induced myokines including irisin, Meteorin-like (METRNL), interleukin 6 (IL-6), lactate and β-aminoisobutyric acid (BAIBA); and 3) insulin as a post-prandially secreted factor. In addition, endocrine stimuli are also visualized including thyroid hormone T3, natriuretic peptides (NP), fibroblast growth factor 21 (FGF21), bone morphogenetic protein 7 (BMP7), bone morphogenetic protein 8b (BMP8b), orexin (OX), vascular endothelial growth factor (VEGF) and prostaglandins (PG). Finally, several synthetic molecules are also shown that can act as BAT inducers but that are no longer used given their adverse secondary effects: mitochondrial uncoupler: 2,4-dinitrophenol, β3-adrenoreceptor agonist: CL 316,243 and PPARγ activators: thiazolidinediones (TZDs). The presented list of factors activating BAT is not exhaustive. A few organ schemes originate from Servier Medical Art. Figure legend: Organs from top (clockwise): Brain,Skeletal muscle, WAT, Heart, Liver, Thyroid, BAT and Pancreas.

Nevertheless, results of adult human-based studies revealed important discrepancies, perhaps due to varying study conditions and experimental designs (10). This illuminates the need for standardized experimental protocols to better assess and compare the actual impact of cold exposure on human BAT activity across individuals and studies.

Exercise

The role of exercise in enhancing and maintaining overall fitness, health and well-being is incontestable. It is an established physiological method for improving muscle strength and cardiovascular system condition, decreasing body weight and allowing its maintenance, protecting against type 2 diabetes, as well as awarding enjoyment through endorphin production (28,29). In light of recent interest in BAT physiology, the cocktail of metabolic factors that is secreted during exercise was analysed in various studies in the context of BAT development and activation (12). This led to the identification of a number of molecules such as Irisin, Meteorin-like, IL-6, lactate, beta-aminoisobutyric acid amongst others (30–35) (Fig. 1). However, as with chronic cold exposure, it remains to be established how much of the beneficial effect of exercise occurs via BAT activation.

Bioactive food ingredients

Another non-drug-based factor discussed in the context of BAT activity enhancement is diet, which along with exercise has a powerful beneficial impact on overall health and fitness. Alternative dietary options, including substances such as green tea, ginger, capsaicin (36) amongst others, have been reported to induce BAT activity. Green tea, for instance, contains caffeine and remarkable quantities of catechin-polyphenols (notably epigallocathechin gallate). These molecules interact to induce 24 h energy expenditure and fat oxidation, thereby implicating them as effective activators of sympathetically mediated thermogenesis (37). Similarly, ginger, with its bioactive constituents gingerols and shogaols (38), was shown to enhance the thermic effect of food (39). Although it was reported that ginger-supplemented high-fat diet fed rats presented lower insulin, glucose and lipids versus the control rats (38), no such effect was observed in an equivalent study conducted in humans (39).

Drug-based approach

The abundance of brown fat and its activation by cold vary according to the extent of obesity of human subjects (7,24,26,40). Studies that directly measure oxidative metabolism in BAT by ^{15}O PET blood flow demonstrated that the level of cold-inducible thermogenesis in most individuals is less than 10 kcal/day (41)–(42). In contrast to cold adapted rats where the blood flow to BAT can reach up to 1,000 ml/min/100 g (43,44), in acute cold-stressed, but chronically warm-adapted humans, the rate is 15–20 ml/min/100 g (41,42,45). To achieve a similar effect, for instance, via exercise, the thermogenic activation of BAT in humans needs to be increased 40–50-fold (46). Hence, drug-based therapies have been proposed to enhance the activity of BAT in metabolically challenged individuals where diet and exercise regimen alone is not sufficient to promote the metabolic activity of BAT. These include chemical uncouplers such as dinitrophenol, which was used extensively but is associated with complications such as hyperthermia and has since been banned. Alternatively, several groups demonstrated the effect of UCP1-specific activators and other futile cycles that may provide metabolic benefit within a tolerable safety profile. This is evidenced by the directed release of mitochondrial protonophores that can improve insulin resistance, diabetes, hypertriglyceridaemia and hepatic steatosis by safely uncoupling in the liver of rats (47). Other drugs include beta-3 selective adrenergic agonist CL-316,243 that can potently activate brown and beige fat in rodents and is known to improve insulin action in human clinical trials (48). Currently, acute dosage of Mirabegron, a drug that has been used to treat an overactive urinary bladder, has been shown to activate brown fat. Mirabegron is therefore a candidate in consideration for a putative drug under the condition that its effect is constant upon chronic dosing and without adverse cardiovascular side effects (49). Thiazolidinediones (TZDs), well-known PPARg agonists, are known to cause browning of white fat by stabilizing PR domain containing 16 (PRDM16), a transcriptional co-regulator of the early brown fat differentiation program. However, they also have a rather negative impact such as weight gain, fluid retention and cardiovascular events, reducing interest in their use. Additional molecules of interest include bone morphogenetic proteins such as BMP7 and BMP8b, cyclooxygenase-2 (COX2) and natriuretic peptides, and fibroblast growth factor 21 (FGF-21). However, these molecules have pleiotropic effects, underscoring the importance of delineating signaling and transcriptional pathways both upstream and downstream of the targeted factor. The following sections will therefore focus on key signaling and gene regulatory modules and mechanisms as well as highlight putative 'druggable' targets.

Part 3: Secreted factors for brown adipose tissue recruitment

Numerous factors were demonstrated to positively recruit BAT (or beige adipose tissue) activity via endocrine, paracrine or autocrine mechanisms (10,50). These include (i) natriuretic peptides that bind to natriuretic receptors on brown or beige adipocytes, and that trigger lipolysis and thermogenesis by activating cyclic GMP-dependent protein kinase (PKG) (10,51); (ii) irisin, a newly identified hormone that is induced in mouse and human muscle by exercise and that stimulates UCP1 expression (10,30); and (iii) orexin, a neuropeptide that triggers the brown fat cell differentiation program by impacting the sympathetic outflow via p38 MAPK as well as the bone morphogenetic protein receptor 1-A-dependent Smad1/5 signaling (10,52) and FGF family members including

FGF-21 and FGF-15/19 that mediate browning and glucose homeostasis (53).

Part 4: Signal transduction pathways

Although lipid-storing cells evolved late during the course of evolution, it has been observed that evolutionarily ancient factors and pathways are highly active during adipogenesis and have either an activatory or inhibitory effect on adipocyte development. Some pathways are also known to exhibit dichotomic effects and are poorly characterized in the context of adipogenesis, particularly regarding how they converge on key transcriptional programs (54). As these signaling pathways control tightly regulated transcriptional cascades, further investigation into the transmission of signals is valuable. The core adipogenic circuit comprises the master regulator PPARg along with CCAAT/enhancer binding protein alpha (C/EBPa), and many signaling events are focused on the activation of this regulatory circuit. On the other hand, initiation of brown fat cell differentiation and activation of the thermogenic circuit require additional transcriptional regulators and co-regulators such as PRDM16, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1a) that are also stimulated by specific signal transduction pathways. In this part, we highlight and summarize important signaling events that impact brown adipogenesis and identify key 'drug-able' targets where possible. A comprehensive overview of key signaling pathways and gene regulatory networks is presented in Fig. 2, highlighting the complexity of the network.

Beta-adrenergic signaling

Beta-adrenergic receptor signaling functions via three types of adrenergic receptors: b, a2 and a1 that respond to the catecholamine, NE which is released as a consequence of cold-induced or leptin-mediated activation of the SNS. The activation of beta-adrenergic receptor signaling further promotes the activation of different signaling pathways in brown adipocytes. Of the three subtypes of beta-adrenergic receptors, beta 3 adrenergic receptor (ADRB3) is predominantly found in mature brown adipocytes, while beta 1 adrenergic receptor (ADRB1) is found in brown pre-adipocytes. Beta-adrenergic receptors are known to couple to G proteins of the Gs subtype and execute the thermogenic signaling cascade via adenylyl cyclase activation. It has been demonstrated that ADRB-1 and -3 mediated thermogenic activation and upregulation of oxidative metabolism are highly effective in elevating whole-body thermogenesis in both rodents (44,55) and humans (56).

Thyroid signaling

Thyroid hormones bind to thyroid hormone receptors (TR) a1–2 and b1–2 to execute pleiotropic effects such as growth and metabolism in several target tissues throughout the body, such as liver, bone, fat and heart. Particularly in brown fat, Thyroxine or T4 is converted to its metabolically active form, Triiodothyronine or T3, by type 2 deiodinase (DIO2) following sympathetic stimulation (57). T3 further acts in concert with norepinephrine to stimulate *Ucp1* gene expression in the BAT in rats (58,59). To determine the TR isoforms regulated by T3, pharmacological and genetic screening approaches have been used, leading to the identification of TRb-1 agonist Gc-1, capable of stimulating *Ucp1* expression but not affecting the regulation of whole body temperature. Further, genetic mouse models showed that TRa activation is important for thermogenesis while TRb is necessary for cholesterol metabolism (60). Thus, drug designs targeting thyroid receptors must take this into account.

Gq signaling

G protein-coupled receptors (GPCRs) are a family of seven transmembrane proteins that regulate biological processes in different tissues such as the adipose tissue, via the action of heterotrimeric G proteins composed of three subunits: alpha (a), beta (b) and gamma (g). Upon activation of GPCRs, Ga dissociates from the Gbg dimer causing the activation of downstream signaling events determined by G protein coupling. Of the four classes of Ga proteins, Gq protein is known to activate multiple intracellular signaling pathways such as ERK, IP3/DAG and RHO/ROCK (61,62). A GPCR profiling-based approach revealed Gq-coupled GPCRs to be one of the highly expressed groups of signaling molecules in murine brown adipocytes that inhibit adipocyte differentiation through the RHO/ROCK signaling cascade. This phenotypic effect was manifested via the Endothelin-1/Endothelin receptor type A (ET-1/EDNRa) signaling axis. Further, overexpression of Gq *in vivo* induced a phenotype similar to the effect of 'whitening' in BAT, preventing 'browning' of WAT in cold-exposed mice (63–65). Given that approximately 25% of the drugs available in the market target GPCRs, it seems feasible to target receptors coupled to Gq to enhance browning in white fat depots (66).

Wnt signaling

Cell fate and development processes are influenced by the para- and autocrine activity of the Wnt family of secreted glycoproteins. The binding of Wnt proteins to frizzled receptors triggers beta-catenin-dependent, canonical signaling pathways. Canonical Wnt signaling ligands such as Wnt10b promote osteogenesis in mesenchymal stem cells (MSCs) and suppress both white and brown adipogenesis. It has previously been demonstrated that Wnt10b blocks brown adipose differentiation and affects the expression of *Ucp1* via repression of *Pgc1a* in mature brown adipocytes leading to a switch to white adipocytes. Wnt signaling

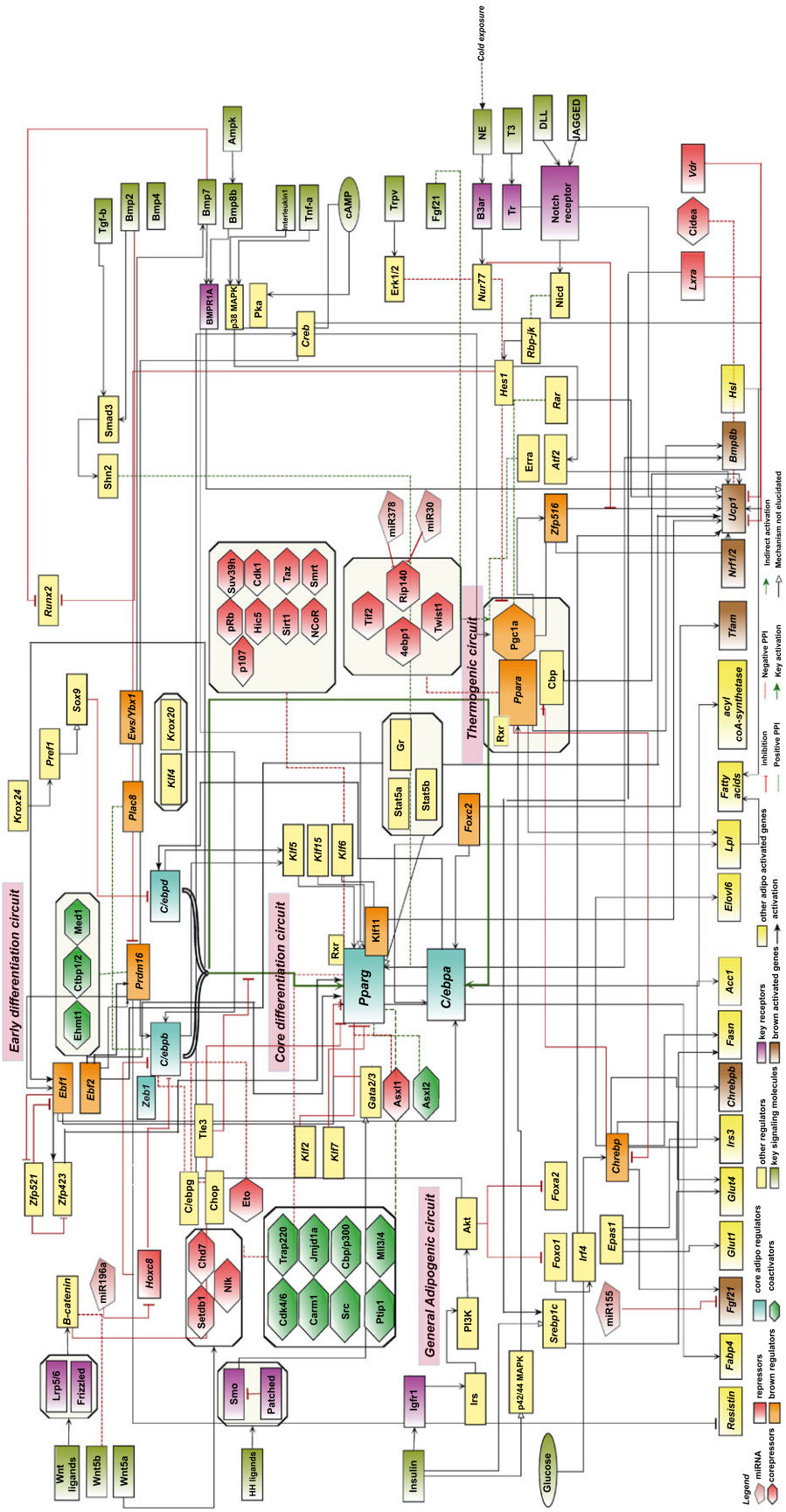

Figure 2 (Continues)

Figure 2 (Continued) Network visualization of key signalling and gene regulatory modules that are implicated in brown adipogenesis. Key signal transduction pathways converging on gene regulatory modules as well as additional transcriptional regulators mediating brown adipogenesis are represented in the network. Nodes in bold italics represent genes, while the ones in bold represent proteins that interact with regulators to control downstream brown-specific and general adipocyte gene expression. The gene regulatory network comprises the following circuits (in chronological order of execution): 1. Early differentiation; 2. Core differentiation and 3. General adipogenic and thermogenic circuit. The network derives from a comprehensive mining exercise of current literature on both general adipogenic and brown signalling and regulatory cascades. Full protein and gene names of the nodes in this network are as follows: *Igfr1*: Insulin-like growth factor 1 receptor, *Irs*: Insulin receptor substrate, *PI3K*: Phosphoinositide 3-kinase, *Akt*: Protein kinase B (PKB), *Lrp5/6*: Low density lipoprotein receptor-related protein, *Hh Ligands*: Hedgehog ligands, *Smo*: Smoothened, *Tgfb*: Transforming growth factor beta, *Bmp2/4/7/8B*: Bone morphogenetic proteins 2/4/7/8b, *Bmpr1A*: Bone morphogenetic protein receptor, type IA, *Smad3*: SMAD, Mothers against DPP homolog 3, *Ampk*: AMP-activated protein kinase, *Shn2*: Schnurri 2, *Tnf-A*: Tumour necrosis factor-alpha *Camp*: Cyclic adenosine monophosphate, *Trpv*: Transient receptor potential (TRP) ion channel, *Fgf21*: Fibroblast growth factor 21, *B3Ar*: Beta adrenergic receptor 3, *Ne*: Norepinephrine, Dll: Delta like, *Nicd*: Notch intracellular domain, *T3*: Triiodothyronine, *TR*: Thyroid receptor, *P42/44 Mapk*: Mitogen-activated protein kinase 1, *Erk1/2*: Mitogen-activated protein kinase, *P38 Mapk*: Mitogen-activated protein kinase, *Pka*: Protein kinase A, *Creb*: cAMP response element-binding protein, *Prdm16*: PR domain containing 16, *C/Ebpb*: CCAAT/enhancer binding protein beta, *C/Ebpd*: CCAAT/enhancer binding protein delta, *Ebf1*: Early B-cell factor 1, *Ebf2*: Early B-cell factor 2, *Zfp521*: Zinc finger protein 521, *Zfp423*: Zinc finger protein 423, *Zeb1*: Zinc finger E-box binding homeobox 1, *Krox24*: Early growth response protein 1 (Egr1), *Pref1*: Preadipocyte factor 1 (aka Dlk1), *Sox9*: SRY (sex determining region Y)-box 9, *Plac8*: Placenta-specific 8, *Ews/Ybx1*: EWS RNA-binding protein 1/Y box binding protein 1, *Runx2*: Runt-related transcription factor 2, Hoxc8: Homeobox C8, *PPARg*: Peroxisome proliferator-activated receptor gamma, *C/EBPa*: CCAAT/enhancer binding protein alpha, *KLF11*: Kruppel like factor 11, *KLF2*: Kruppel like factor 2, *KLF7*: Kruppel like factor 7, *KLF5*: Kruppel like factor 5, *KLF15*: Kruppel like factor 15, *KLF6*: Kruppel like factor 6, *Rxr*: Retinoid X receptor, *Gata2/3*: Globin transcription factor 2/3, *Epas1*: Endothelial PAS domain protein 1, *Chrebp*: Carbohydrate response element binding protein, *Srebp1c*: Sterol regulatory element binding transcription factor 1, *Foxo*: Forkhead box O1, *Foxa2*: Forkhead box A2, *Irf4*: Interferon regulatory factor 4, *Erra*: Estrogen-related receptor alpha, *Ppara*: Peroxisome proliferator-activated receptor alpha, *Cbp*: CREB binding protein, *Zfp516*: Zinc finger protein 516, *Foxc2*: Forkhead box C2, *Tle3*: Transducin-like enhancer of split 3, *Vdr*: Vitamin D receptor, *Lxra*: Liver X receptor alpha, *Rbp-Jk*: Recombination signal binding protein for immunoglobulin kappa J, *Hes1*: Hes family BHLH transcription factor 1, *Nur77*: Nuclear hormone receptor NUR/77, *Atf2*: Activating transcription factor 2, *Rar*: Retinoic acid receptor, *C/ebpg*: CCAAT/enhancer binding protein gamma, *Chop*: C/EBP-homologous protein 10, *Ehmt1*: Euchromatic histone-lysine N-methyltransferase 1, *Ctbp1/2*: C-terminal binding protein ½, *Med1*: Mediator complex subunit 1, *Eto*: Runt-related transcription factor 1; translocated to, 1 (cyclin D related), *Setdb1*: SET domain, bifurcated 1, *Chd7*: Chromodomain helicase DNA binding protein 7, *Nlk*: Nemo like kinase, *Asxl1*: Additional sex comb like 1, *Asxl2*: Additional sex comb like 2, *p107*: Retinoblastoma-like 1, *pRb*: Retinoblastoma protein, *Suv39*: Suppressor of variegation 3–9 Homolog 1, *Hic5*: Hydrogen peroxide-inducible clone 5 protein, *Cdk1*: Cyclin-dependent kinase1, *Sirt1*: Sirtuin 1, *Taz*: Transcriptional coactivator with PDZ-binding motif, *NCoR*: Nuclear receptor corepressor 1, *Smrt*: Silencing mediator for retinoid and thyroid hormone receptors, *Tif2*: Transcriptional intermediary factor 2, *4ebp1*: Eukaryotic translation initiation factor 4E-binding protein 1, *Rip140*: Nuclear receptor interacting protein 1, *Twist1*: Twist family BHLH transcription factor 1, *Pgc1a*: Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha, *Cidea*: Cell death-inducing DFFA-like effector A, *Stat5a*: Signal transducer and activator of transcription 5a, *Stat5b*: Signal transducer and activator of transcription 5b, Gr: Glucocorticoid receptor, *Tfam*: Transcription factor A, mitochondrial, *Nrf1/2*: Nuclear respiratory factor ½, *Ucp1*: Uncoupling protein 1, *Hsl*: Hormone-sensitive lipase, *Lpl*: Lipoprotein lipase, *Fabp4*: Fatty acid binding protein 4, *Fgf21*: Fibroblast growth factor 21, *Glut1*: Glucose transporter type 1, *Glut4*: Glucose transporter type 4, *Irs3*: Insulin receptor substrate 3, *Chrebp*: Carbohydrate response element, *Fasn*: Fatty acid synthase, *Acc1*: Acetyl-CoA carboxylase Alpha, *Elovl6*: ELOVL fatty acid elongase 6.

may suppress *Pgc1a* either directly via a beta-catenin LEF1 complex, or interference with transcriptional activity of *Pgc1a* regulators such as ATF-2, CREB, FOXO1 or via the inhibition of upstream signaling molecules such as protein kinase A or p38 MAPK (67). Other Wnts such as Wnt6, Wnt10a are also known to suppress adipogenesis and promote osteoblastogenesis (68). In contrast, Wnt5b positively regulates adipogenesis by countering the nuclear translocation of beta-catenin, thus inhibiting the canonical Wnt signaling pathway (69).

Hedgehog signaling

Hedgehog (HH) ligands bind to the Patched-1 (PTCH1) receptor and liberate Smoothened (SMO), a downstream protein in the pathway, from the inhibitory effect of PTCH1 causing SMO to regulate gene transcription of GLI proteins that negatively regulate adipogenesis (54). Also, it is likely that SMO indirectly activates the GATA factors that are known to be anti-adipogenic (70). Extrapolation of results from a systems level genetic dissection of adipose regulation in *Drosophila melanogaster* to the murine system implicated HH signaling pathway as being inhibitory for white but not for brown fat formation (71). In contrast, a more recent study demonstrated that HH signaling operates in a cell-autonomous manner to block brown adipocyte differentiation, inhibit *in vivo* BAT formation and replace neck BAT in rodents into poorly differentiated skeletal muscle, partially via the upregulation of chicken ovalbumin upstream promoter transcription factor II (Coup-TFII) (72). The differences between these two studies could be attributed to the *in vivo* system used to assess the phenotypic effect of HH signaling on adipose depots thereby highlighting the importance of interpreting phenotypic outcomes obtained from *in vivo* data (72).

Janus kinase–signal transducers and activators of transcription signaling

Janus kinases (JAKs) are associated with various cell surface receptors, whose activity is triggered via ligands such as interferon, interleukin and growth factors. Activated JAKs recruit SH2 domain containing proteins (signal transducers and activators of transcription (STATs)) to the receptors via phosphorylation and are further tyrosine phosphorylated by JAKs. These activated STATs then translocate to the nucleus, form hetero- or homo-dimers and regulate transcription of target genes. Recently, Janus kinase inhibitors were identified as molecules that possess 'browning' potential, using a screening approach to discover small molecules capable of promoting a switch from white to brown in human adipocytes. It was demonstrated that two compounds, tofacitinib and R406, strongly induce *UCP1* while remodeling lipids via the JAK–STAT1/3 pathway, thereby implicating the IFN–JAK–STAT–SHH axis in human adipocyte biology. Of the two potential drugs identified, the drug

tofacitinib is sanctioned in the United States for rheumatoid arthritis (73). Additionally, because the JAK–STAT pathway is involved in immune system regulation, local administration of the drug or *ex vivo* transplantation of cells post-treatment is recommended to limit deregulated immune activation.

Transforming growth factor-beta signaling

Transforming growth factor beta (TGF-b), myostatin and bone morphogenetic proteins regulate the differentiation of many cell types including adipocytes. While subsequent signaling events have common properties, specific TGF-b family members influence distinct cell fate decisions by binding to serine/threonine kinase receptors and functioning through SMAD-dependent and -independent mechanisms. Transcriptional regulation of target genes is executed by receptor-regulated SMAD1 and SMAD3 TFs, which, upon phosphorylation, engage in dimer formation with SMAD4, and subsequently translocate to the nucleus. In adipogenesis, the canonical TGF-b signaling pathway has an unclear molecular function. While TGF-b expression correlates positively with obesity in humans, it inhibits *in vitro* adipogenesis of 3T3-F442A cells by signaling through SMAD3. It has been observed that supplementing wild-type mice with exogenous TGF-b1 reduced thermogenic gene expression in fat (74), implicating TGF-b signaling in the negative regulation of browning. This has been exploited for therapeutic purposes as evidenced in a study where a dominant negative form of activin receptor type II-b fusion protein was used to promote thermogenesis in mice by binding to TGF-b, thereby preventing downstream signaling (75).

Bone morphogenetic protein signaling

Bone morphogenetic proteins (BMPs) are categorized as a family within the TGF-b superfamily with the ability to stimulate white and brown fat cell differentiation programs. While BMP2 and BMP4 are known to induce commitment to the white adipocyte fate when supplemented by a hormonal induction cocktail, BMP7 is known to singularly support brown fat cell differentiation in both MSC progenitors and committed pre-adipocytes even in the absence of a differentiation cocktail via the p38 MAPK pathway (76). p38 MAPK further executes the thermogenesis program by phosphorylating and activating distinct nuclear receptors (NRs), ATF2 and PGC1a, which in turn promote *Ucp1* expression by binding to a cAMP and PPAR-response element that resides in a key enhancer of the *Ucp1* gene (77). This phenotypic effect was also observed *in vivo*, because *Bmp7* null mice show a reduction in brown fat mass that was rescued upon adenovirus-mediated overexpression of Bmp7, thereby protecting the mice from diet-induced obesity while triggering energy expenditure (76). BMP7 is also known to function synergistically with rosiglitazone, a TZD, to give rise to inducible brown adipocytes from SCA1+ adipocyte progenitor cells that arise from different fat depots as well as skeletal muscle in mice (78). Additionally, *in vivo* studies showed that BMP7 enhances *Ucp1* expression in WAT in combination with the beta3-adrenergic agonist CL-316,243 to levels higher than those achieved by CL-316,243 treatment alone (78). This study underscores the importance of the BMP7 signaling pathway in inducing browning and as a viable therapeutic option.

BMP8b is yet another BMP signaling molecule capable of regulating whole body energy metabolism both centrally, through AMPK activation in hypothalamic nuclei, and peripherally, by activating the p38 MAPK signaling pathway in differentiating and mature brown adipocytes. Using *Bmp8b* knockout mice, it was demonstrated that BMP8b increases the peripheral response of BAT to adrenergic stimulation and acts centrally to increase sympathetic output to BAT. BMP8b signaling specifically acts on the thermogenic pathway, as *Bmp8b* knockout mice do not show gross abnormality in BAT morphology. Additionally, it has been proposed that AMPK has a counter-regulatory role, acting in opposition to BMP8b to regulate energy expenditure, supported by the observation of greater thermogenic activation upon localized expression of the dominant negative AMPK alpha isoform. As AMPK is a known druggable target in peripheral tissues to treat insulin resistance (79), its relationship with BMP8b offers new opportunities in targeted drug design. It is worth noting that the ability of BMP8b to execute central actions to reduce whole body weight without compensatory food intake offers an exciting premise for new therapeutic options, demonstrating the importance of understanding thermogenesis at a central regulatory level.

NOTCH signaling

The Delta-like (DLL) and Serrate/Jagged (JAG) family of membrane bound ligands bind to transmembrane NOTCH receptors causing gamma-secretase-mediated proteolytic cleavage of NOTCH, leading to the nuclear translocation of the NOTCH intracellular domain (NICD). Once in the nucleus, the NICD activates the RBP-jk transcriptional complex and subsequently its downstream targets such as the *Hes* and *Hey* family genes (80). Constitutive activation of NOTCH signaling inhibits *Pgc1a* and *Prdm16* transcription in white adipocytes via HES1-mediated transcriptional repression and causes 'whitening' of BAT. In contrast, inhibition of NOTCH signaling is associated with 'browning' of WAT, improvement of insulin sensitivity and whole-body energy homeostasis (81). This differential response is intriguing and highlights the involvement of different gene regulatory programs that are cell-context dependent. As NOTCH signaling is an evolutionarily conserved pathway, these findings can be extended to human studies; however, this merits further investigation into identifying molecular

mechanisms operating in human white to brown conversion.

Part 5: Gene regulatory networks

Once adipogenic precursors commit to the adipogenesis program, a transcriptional circuit is switched on, leading to the expression of genes associated with fat cells. While the white and brown differentiation programs share a set of regulators and adipogenic stimuli, they both have unique requirements that determine their phenotypic differences. It is therefore important to delineate both, i.e. the core adipogenic differentiation and the thermogenic programs, to get a full picture of the gene regulatory events that give rise to fully functional brown and brite fat. In this section, we present an overview of the individual circuits such as the early and core differentiation, thermogenic and adipogenic modules within the overall gene regulatory network. A schematized overview is presented in Fig. 2.

Early differentiation circuit

Early B-cell factors

The early B-cell factors (EBF), especially EBF1 and EBF2, play an important regulatory function in the early differentiation circuit along with Zinc Finger Protein (ZFP) 423 and its close paralog ZFP521, both implicated as early regulators of adipose commitment and differentiation (82,83). ZFP521 functions in a repressive feedback loop with EBF1, while also repressing ZFP423, thereby influencing early regulatory events in the lineage switch between bone and fat (83). ZFP423, on the other hand, functions by promoting *Pparg* expression through amplification of the BMP signaling pathway via SMAD binding (82). Further, *Ebf1* is induced by both CCAAT/enhancer binding protein delta (C/EBPd) and CCAAT/enhancer binding protein beta (C/EBPb) and then directly activates both *Pparg* (via binding to the *Pparg1* promoter) and *C/ebpa*, which positively feeds back to *C/ebpd* (84). A previous study demonstrated EBF2 to regulate PPARg binding to determine brown versus white fat fate, leading to its classification as a brown-specific transcriptional regulator (85). It was further suggested that BAT-specific target genes of PPARg were categorically bound by EBF2 that then served as a mark for the later recruitment of PPARg at sites regulating the expression of brown fat selective genes such as *Prdm16* and *Ucp1* (85).

PR domain containing 16

The 140 kDa PRD1-BF-1-RIZ1 homologous domain containing protein-16, PRDM16, is a core transcriptional co-regulator in both brown and beige fat, showing selective expression in brown over white fat (13). *In vitro* cell culture studies in classical brown fat cells demonstrated the requirement of PRDM16 to develop and maintain a thermogenic gene program. However, *in vivo* deletion of *Prdm16* did not affect the function of classical brown fat but had a significant impact on the development of beige fat cells. Additionally, deletion of *Prdm16* along with its closest homolog *Prdm3* led to an early and extreme loss of brown fat thermogenic gene expression (86). Most importantly, PRDM16 has been demonstrated to function as a molecular switch between white and brown programs by coupling with diverse protein interaction partners simultaneously, mediating both activating and repressive gene expression programs. These dichotomous activities are executed through the use of chromatin remodellers, C-terminal binding proteins (CtBPs) that function via the recruitment of histone deacetylases (87). PRDM16 interacts with either PGC1a or CtBPs to, respectively, activate the brown or suppress the white gene expression program. During brown fat activation, recruitment of PGC1a displaces CtBPs from the complex to activate brown fat-specific genes. Additionally, it has been suggested that PRDM16 binds to specific TFs linked to the promoters/enhancers of target genes, further guiding the recruitment of PGC1a or CtBPs to initiate the formation of a co-activator or a co-repressor complex (87). The TFs that guide PRDM16 to its respective target genes and their molecular underpinnings have not been mapped yet, and it is quite likely that numerous factors are involved in this regulatory process.

Placenta-specific 8

Placenta-specific 8 (PLAC8), a key upstream molecule in the brown adipogenic regulatory network, functions by associating with *C/ebpb* and binding to its promoter to induce transcription (88). *In vivo* genetic deletion of *Plac8* results in the loss of expression of several brown fat-specific components such as *Prdm16* and *Pgc1a*. However, general adipocyte differentiation factors such as PPARg and AP2 are not downregulated, indicating the importance of PLAC8 in recruiting BAT during thermogenic challenges. Consequently, *in vivo* genetic inactivation of *Plac8* leads to errors in BAT differentiation and thermogenic programs, causing cold intolerance and late onset obesity (88). However, the upstream signaling activators of *Plac8* induction and those that promote PLAC8 binding to C/EBPb are as yet uncharacterized and merit further investigation.

CCAAT/enhancer binding protein beta and CCAAT/enhancer binding protein delta

PPARg and C/EBPs, the key molecular players of adipogenesis, vary in their molecular function in brown versus white fat programs. In response to pro-adipogenic stimuli, C/EBPb and C/EBPd transition from a repressive complex, involving the nuclear co-repressors NCoR1 and SMRT (89), into an active one. This in turn allows these TFs to induce expression of PPARg and C/EBPa in cooperation

with the epithelial–mesenchymal transition TF ZEB1 (90), which drives terminal fat cell differentiation. C/EBPb is important for adipogenic differentiation in immortalized adipocytes with slightly inconspicuous effect in mouse embryonic fibroblasts (MEFs). While it has been demonstrated that C/EBPb-deficient mice have reduced adiposity, this might be attributed to impairment in lipogenic and not adipogenic programs (54,91). It is quite likely that C/EBPd may take over for C/EBPb in a knock out model, as a double knockout mouse model for *C/ebpb* and *C/ebpd* shows a further decline in adipose tissue mass (91). Moreover, C/EBPb has been shown to work in collaboration with PPARg to induce the expression of *C/ebpa,* as PPARg is required to release the inhibitory chromatin remodeler HDAC1 from the *C/ebpa* promoter (92). Thus, C/EBP -b and -d work synergistically with other members of the core differentiation circuit to execute downstream gene regulatory programs.

Core differentiation circuit

Peroxisome proliferator-activated receptor gamma

The ligand-dependent nuclear receptor PPARg expressed primarily in adipocytes is induced during adipogenesis and is central to the adipogenic regulatory network. It has two different isoforms, PPARg1 and PPARg2, of which PPARg2 is exclusively present in the adipose tissue in contrast to PPARg1, which is ubiquitously expressed. PPARg functions as a regulator in adipogenic programs common to both white and brown fat differentiation, and no other factor has so far been found that can promote adipogenesis in the absence of PPARg. All pro-adipogenic transcriptional regulators such as C/EBPs and some KLFs such as KLF5, KLF15 converge on one of the two *Pparg* promoters while anti-adipogenic transcriptional regulators such as the GATA factors function by repressing *Pparg* expression (93–95). Additionally, key adipogenic signaling pathways also converge on *Pparg* expression and activity. Upon activation by ligands, PPARg forms a heterodimer with RXR and binds to a Ppar response element (PPRE) in cooperative fashion (96,97), enabling it to control the expression of a specific subset of PPRE-containing genes such as *Ap2*, *Fabp4* and *Lpl* in white adipocytes (98). Studies showed that stimulation of white adipocytes *in vitro* or *in vivo* by strong PPARg agonists such as rosiglitazone leads to 'browning' of the white cells (99). The latter triggers an upregulation of mitochondrial genes such as *Ucp1*, *Cox7a1* and *Cox8b*, subsequently causing an increase in mitochondrial mass, enhanced oxygen consumption and lipid oxidation (100,101). This browning effect is characterized by a suppression of adipokine production such as Resistin, Alpha1-acidoglycoprotein and Haptoglobin (102–104). The phenotypic outcome of supplementing white adipocytes already known to express *Pparg* and its endogenous ligands with additional PPARg agonists suggests that brown adipocytes produce a distinct repertoire of PPARg ligands that differ in activity from those produced in white adipocytes (105). It is likely that this regulatory mechanism is then executed via the actions of PRDM16/PGC1a and PRDM16/CtBP complexes as they are known to mediate brown versus white programs.

CCAAT/enhancer binding protein alpha

C/EBPa plays a crucial role in differentiated adipocytes and works in concert with PPARg to constitute the core differentiation circuit. The molecular function of C/EBPa is central to the acquisition of insulin sensitivity as opposed to accumulation of lipids or expression of adipocyte genes (106).

Nuclear Factor IA

The Nuclear Factor I (NFI) family of TFs including NFIA, NFIB, NFIC and NFIX are widely expressed, with each TF regulating a distinct set of target genes as evidenced by different phenotypes yielded by mouse knockout models (107). Previously, both NFIA and NFIB have been identified as positive regulators of white adipocyte differentiation via *in vitro* cell culture studies in the 3T3L1 cell line (108). Using an integrative genomics approach, we discovered that NFIA is also involved in the brown adipogenesis gene regulatory network (109). Notably, we observed that *Nfia* is differentially expressed during brown fat cell differentiation and has a strong positive correlation with *Ucp1* expression levels in the mouse genetic reference panel (BXD) (110) and human clonal BAT cell lines (111). Further, genome-wide localization analyses based on ChIP-seq revealed that, while NFIA does not directly bind to *Ucp1*, it does, however, bind to general adipogenic marker genes such as *Zfp423*, core adipogenic regulators such as *Pparg* and *Rxrg*, early brown specific TFs such as *Ebf2* and finally drivers of the thermogenic module such as *Ppara* and *Pgc1a*. A follow-up on the mechanistic details of NFIA action in brown versus white is necessary, especially to delineate its mode of regulatory action in these tissue types.

Apart from the core members of this differentiation circuit, additional adipogenic regulators include positive regulators such as ZEB1 (90), ASLX2 (112), Kruppel like factors (KLF)-5 (93), -15 (113) and 6 (114), STAT5a (115,116), while negative regulators include GATA transcription factors (94,95), ASLX1 (112), KLF-2 (117). These transcriptional details have been reviewed extensively elsewhere (54,105).

Thermogenic circuit

Peroxisome proliferator-activated receptor alpha

The peroxisome proliferator-activated receptor alpha (PPARa), a protein from the nuclear receptor family comprising PPARg/d/b, is a fatty acid-activated TF that executes transcriptional regulation of genes involved in

cellular metabolism (118). It is particularly detected in tissues such as brown fat, liver or heart, known to have high rates of fatty acid oxidation and peroxisomal metabolism. High expression of *Ppara* distinguishes brown from white fat and is related to the lipid oxidation capacity of brown fat. It was shown that PPARa activators stimulate *Ucp1* gene expression both in brown adipocytes and mature BAT, acting through a PPRE element situated in the upstream enhancer of the *Ucp1* gene that is also responsible for PPARg-dependent regulation (119). PPARa interacts with CBP and PGC1a, forming a tight regulatory complex to activate *Ucp1* gene expression. An interesting regulatory mechanism exists between the two PPARs, PPARa and PPARg, during brown adipogenesis. While it was demonstrated that PPARa and PPARg bind and activate *Ucp1* gene transcription through the same Ucp1-PPRE element, the predominant molecular function of any particular subtype depends on the relative amount of each subtype. For example, crosstalk with other signaling pathways such as regulation of PPAR transcriptional activity by MAPK-dependent phosphorylation enhances PPARa (120) but decreases PPARg activity (121), as well as ligand availability and interaction with co-regulators such as PGC1a. The interaction of PGC1a with PPARa is ligand dependent, whereas the one with PPARg is not (122). Therefore, these and possibly other uncharacterized events may determine which PPAR subtype activates *Ucp1* transcription in response to brown adipocyte-inducing conditions. In other words, it could be PPARg in association with differentiation-dependent events, or PPARa in conjuction with increased lipid catabolism in active BAT, highlighting the importance of understanding this regulatory mechanism. Further, it was observed that acute administration of the PPARa ligand Wy 14,643 upregulates *Ucp1* mRNA expression in those physiological situations in which endogenous PPARa ligands are expected to be low. This is in agreement with previous findings demonstrating that PPARa sensitivity *in vivo* depends upon the status of lipid metabolism (123).

Carbohydrate response element binding protein

Yet another interesting mechanism is the differential regulation of PPARa and carbohydrate response element binding protein (ChREBP), a TF that is responsive to glucose signaling and is more highly expressed in brown compared to white fat tissue, influencing lipid storage and lipolysis in brown adipocytes. It was demonstrated that ChREBP and PPARa regulate each other via an inhibitory feedback mechanism that is activated during conditions of high and low glucose, respectively (124). As such, ChREBP and PPARa coordinately establish a feedback loop between lipogenesis and lipolysis in brown adipocytes. The molecular mechanism underlying this crosstalk remains largely unexplored and may serve as an avenue for further research to develop novel therapeutics.

Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha

Given that PPARg alone is insufficient to induce browning, extensive investigation into other putative regulators led to the identification of PGC1a. The involvement of PGC1a was first demonstrated in the thermogenic circuit when a dramatic increase in *Pgc1a* expression was observed in mice exposed to cold in both skeletal and brown fat (122). PGC1a is also triggered in brown fat cell lines upon treatment with the beta-adrenergic receptor agonist isoproterenol (122). It interacts with multiple nuclear hormone receptors that bind to the *Ucp1* gene enhancer in either a ligand-dependent or -independent manner as in the case of Retinoic Acid Receptor (RAR) and Thyroid Receptor (TR) or PPARg, respectively. PGC1a not only boosts the efficiency of transcription but also plays a key role in specifying the genes that are targeted by its partner TFs. This is illustrated by overexpression of PGC1a in white fat cells leading to the induction of *Ucp1* through the PPARg binding sites but a failure to express the PPARg target gene *Ap2*. As PGC1a is a co-activator and is dependent on the binding of particular TFs to implement different gene expression programs, repression of binding events could affect different biological responses. Thus, targeting singular binding events between PGC1a and particular TFs might offer new therapeutic opportunities.

However, while PGC1a is a critical regulator of adaptive thermogenesis, it is not the master regulator of brown differentiation. This is evidenced by the fact that reduction in PGC1a levels during brown fat cell differentiation has no significant impact on brown adipogenesis. However, it has a strong impact on the induction of thermogenic genes by cAMP (105). Apart from its molecular involvement in regulating thermogenesis, it also aids in inducing angiogenesis via the co-activation of the orphan nuclear receptor ERRA on the *Vegf* promoter. PGC1a was stimulated by an inadequacy in nutrients as well as oxygen leading to increased *Vegf* expression and subsequently angiogenesis in BAT and skeletal muscle *in vivo* (125). Thus, the transcriptional functions of PGC1a are key to the development and thermogenic activity of brown adipocytes.

Forkhead box C2

Forkhead box protein C2 (FOXC2), a member of the Forkhead box family of TFs, regulates different aspects of adipocyte metabolism by activating PPARg and C/EBPa as well as by enhancing cAMP-mediated PKA signaling (126). Most importantly, FOXC2 responds strongly to high caloric load via strong induction of cAMP signaling as demonstrated by high fat feeding experiments in which FOXC2-overexpressing mice showed reduced weight gain in response to the amount of food consumed. This categorizes FOXC2 as a putative therapeutic target based on its molecular function as a metabolic regulator and a factor that can induce adaptive thermogenesis (126). Additionally,

enhanced expression of *Foxc2* has also been implicated in mitochondrial biogenesis via trans-activation of the nuclear encoded mitochondrial transcription factor A as well as induction of mitochondrial fusion via activation of Mitofusin 1 and 2, and Optic atrophy 1 homolog (127). This correlation was observed in humans, making FOXC2 an attractive target for therapy. *Foxc2* expression was also shown to increase in response to high caloric feeding, whilst repressed by cold (127). This suggests that FOXC2 has a distinct role in regulating mitochondrial function from that observed in cold-adapted thermogenesis, known as metabolo-regulatory thermogenesis. This is different from thermo-regulatory thermogenesis, which is categorically induced by cold and is dependent on expression of *Pgc1a* and *Ucp1* (8).

In addition to the key factors described above, new factors have recently been implicated in early brown adipogenesis. These include the Kruppel like factor 11 that binds to PPARg and activates brown fat genes in rosiglitazone-treated human adipocytes (128) as well as the EWS/YBX1/BMP7 axis, in which the RNA-binding protein EWS along with its partner YBX1 transcriptionally activates *Bmp7* to induce browning (129). Additionally, new factors implicated in the thermogenic program are Zinc-finger protein 516 (ZFP516) that is involved in the activation of *Ucp1* gene expression by interacting with PRDM16 (130) and interferon regulatory factor-4 (IRF4), an important TF partner of PGC1a that both induces and interacts with PGC1a to activate thermogenic genes (131). A new regulatory layer comprising small non-coding RNAs, miRNAs such as miR-193b-365, miR-133, miR-196a, miR-155, miR-27, miR-378, miR-34a along with BAT-specific long-noncoding RNAs such as lnc-BATE1 and Blnc1 is also known to promote brown and/or beige adipocyte differentiation and physiological function (132).

Anti-adipogenic regulators affecting the PGC1a–UCP1 axis

Given the central function of PGC1a and UCP1 in the thermogenic program, their expression and activity are tightly regulated by a multitude of factors that serve as negative regulators. This mode of negative regulation most likely functions to restrain thermogenesis, whose prolonged activation may lead to adverse conditions such as hyperthermia, which in turn has the potential to cause organ failure and death (133).

Nuclear receptor interacting protein 140

Nuclear receptor (NR) interaction protein 140 (Rip140) is a ligand-dependent transcriptional repressor of nuclear receptors including estrogen receptor and PPARs and also functions as a transcriptional co-repressor that shares a number of downstream targets with PGC1a and inhibits PGC1a's transcriptional activity by binding to it directly (134). Along with suppressing PGC1a target genes, Rip140 also represses *Ucp1* expression by recruiting inhibitory histone modifying and DNA methylation enzymes to the promoter of *Ucp1* (135). Lack of Rip140 increases *Ucp1* expression by de-repression of PPARa, PPARg and PGC1a. Studies suggest that, given Rip140's high expression in WAT compared to BAT, it functions to suppress the brown phenotype. This is evidenced by significant enhancement of brown-related genes upon knockdown of *Rip140* in white fat cells (136). Further, observations from *in vivo* studies demonstrated that *Rip140* null mice exhibit expenditure rather than storage of the consumed fat (137). However, in-depth investigation into the molecular mechanisms underlying Rip140-mediated 'browning' suppression is pending.

Liver X receptor alpha

The Liver X receptor alpha (LXRa) does not directly interfere with PGC1a's transcriptional activity or binding, rather, when ligand-activated, this TF interferes with the induction of *Ucp1* expression by displacing PPARg from the *Ucp1* enhancer and by drafting RIP140 as a corepressor to its binding site (133). It has also been demonstrated that mice lacking LXRa show increased *Ucp1* expression in both BAT and WAT with no significant change in the expression of *Pgc1a*, thus manifesting a lean phenotype (133,138).

Retinoblastoma protein

pRb acts as a molecular toggle between the white and brown adipocyte differentiation programs (139) by direct binding to the *Pgc1a* promoter leading to transcriptional repression (140). Additionally, pRb functions as a PPARg co-repressor, thereby suppressing its transcriptional activity (141).

Twist basic helix–loop-helix TF 1

Twist basic helix–loop-helix TF 1 (TWIST1) functions as an antagonist of brown fat-mediated thermogenesis by binding directly to PGC1a and suppressing its transcriptional activity. This is executed by Twist1-mediated recruitment of the histone deacetylase HDAC5 to the promoters of PGC1a target genes, causing transcriptional repression (142).

Other key anti-adipogenic factors suppressing the PGC1a and UCP1 axis include unliganded Vitamin D receptor that directly suppresses *Ucp1* expression via binding to a Vitamin D receptor element in the proximal region of the *Ucp1* promoter (133), steroid receptor co-activators such as SRC2 and SRC3 that repress the transcriptional activity of PGC1a, TRPV4 (a transient receptor potential cation channel 4) that represses PGC1a via the ERK1/2 protein kinases (143) and SHP (orphan nuclear receptor) that inhibits the ERR-g mediated promoter transactivation of PGC1a (133).

Outlook

While the white adipocyte differentiation network is well characterized, the brown adipogenic signaling and gene regulatory network remains rather sparse, warranting further investigation to annotate missing nodes and interactions. The latter include additional regulators that are capable of inducing browning in concert with key molecular players, thereby providing alternative points of safe therapeutic intervention. Most notably, through a comprehensive literature-based reconstruction of the brown signaling and gene regulatory network, we illustrate the requirement to focus on delineating implicated signaling pathways and on how they converge on the transcriptional components. This will enable a more thorough understanding of key molecular events and guide better drug discovery efforts. An additional focus should be on elucidating mechanisms that govern the white to brown switch, aiding in the development of drugs that can safely and specifically mediate browning.

Recent studies have also elucidated alternative modes of thermogenesis that function without the need for UCP1 activation, wherein beige and white adipocytes can directly respond to cool temperature (27–33°C) and activate a thermogenic program in a cell-autonomous manner. This particular type of thermogenic program was determined to be independent of the canonical cAMP/protein kinase/CREB pathway downstream of beta-adrenergic signaling (144). Furthermore, in a study conducted on human subjects, it was observed that elevated thermogenesis upon cold exposure is not inhibited by the chemical blockade of beta-adrenergic signaling alone (145). This suggests the existence of alternative pathways for induction of a thermogenic program through a cell-autonomous effect of temperature on certain fat depots. Recently, a futile cycle of creatine metabolism has been identified as an alternative pathway for energy expenditure and thermal homeostasis (146). The implicated proteins Creatine Kinase Mitochondrial 1 and 2 (CKMT1 and CKMT2) were enriched in human BAT, raising the possibility of exploiting this pathway in manipulating energy consumption in patients with metabolic diseases, particularly via dietary supplementation and/or administration of novel drugs.

One of the main challenges in the field of translational research is the design of safe therapeutic interventions. Several treatment options targeting BAT are being explored, but more often than not, they suffer from potential systemic side effects. We believe that a systems perspective summarizing various input stimuli, downstream signaling and transcriptional components will provide a strong framework to aid in the development of targeted drug design. Furthermore, the field's focus should not only be on identifying specific druggable pathways but also strategies for drug delivery such as the development of controllable microenvironments, scaffolds, biomaterials for transplantation of energy expending cells as well as focused pharmacological intervention to stimulate the activity of resident progenitor cells *in situ* by triggering endogenous brown adipogenesis.

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Conflict of interest statement

All authors declare no conflict of interest.

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